Competition at silent synapses in reinnervated skeletal muscle

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Synaptic connections are made and broken in an activity-dependent manner in diverse regions of the nervous system. However, whether activity is strictly necessary for synapse elimination has not been resolved directly. Here we report that synaptic terminals occupying motor endplates made electrically silent by tetrodotoxin and α-bungarotoxin block were frequently displaced by regenerating axons that were also both inactive and synaptically ineffective. Thus, neither evoked nor spontaneous activation of acetylcholine receptors is required for competitive reoccupation of neuromuscular synaptic sites by regenerating motor axons.

Neural development and repair include stages in which neurons make more connections with their target cells than they ultimately retain in the normal adult[1,2]. This initial excess and subsequent decrease in neuronal divergence is matched, from the perspective of the postsynaptic cell, by a decrease in convergence; that is, the numbers of connections that each neuron or target cell receives also decreases during postnatal development or after nerve regeneration. For example, during the development of the mammalian visual system, the distribution of connections from the thalamus to layer 4 of primary visual cortex is transformed from a diffuse pattern to an ordered, alternating pattern of ocular dominance. In neonatal rat cerebellum, individual Purkinje cells are initially innervated by several climbing fibers, but these inputs are pared away until only one remains[1]. Changes in the number and disposition of connections occur in other parts of the nervous system, including the olfactory bulb, hippocampus, spinal cord and autonomic ganglia[3]. In most cases, these developmental changes involve a decrease in overlap of neural connectivity.

These changes in neuronal divergence and convergence are generally considered to be neither spontaneous nor random. Rather, they seem driven by competition for as-yet-unidentified molecular resources, leading to selective growth of some synapses and elimination of others. There is overwhelming evidence that patterns of use or disuse of connections strongly bias the rate and outcome of competitive synapse elimination. However, whether experience or activity are strictly necessary for the induction or expression of synapse elimination has not been satisfactorily resolved. For example, the initial organization of connections in the visual system is reported not to require either visual experience[4,5] or neural activity[6]. In hippocampal neurons, strengthening of groups of synaptic connections may occur locally at electrically stimulated sites and at nearby, unstimulated sites[7,8].

Neuromuscular connectivity offers a tractable model system in which to investigate the importance of activity in sculpting innervation patterns at the level of individual, identified synapses. Synaptic boutons converge on single motor endplates, domains defined by unique structural specializations (junctional folds) and a high density of acetylcholine receptors and other synapse-specific proteins[9]. Hyperinnervation (polyneuronal innervation) of muscle fibers occurs during development[10]. Polyneuronal innervation is transformed to mononeuronal innervation as convergent synapses are competitively eliminated from the motor endplate areas. Similar changes occur after nerve injury, because of divergence of intact motor units through axonal sprouting and regeneration of injured motor axons to endplates occupied by sprouts[11]. The transformation from polyneuronal to mononeuronal innervation is delayed by neuromuscular paralysis and resumes when activity is restored[12]. Differences in the activity of convergent synapses confer competitive advantages on the more active[13–15]. These findings suggest that competitive neuromuscular synapse elimination is strongly influenced by endogenous activity. However, many observations do not agree with a decisive function for activity in synapse elimination. For example, some polyneuronal junctions persist in the presence of activity[12,16] and, conversely, sometimes inactive synapses have a competitive advantage over active ones[11,17,18]. We therefore set out to stringently test the hypothesis that there is an absolute requirement for activity in competitive synapse elimination in reinnervated muscle.

Our experimental design took advantage of the dual motor nerve supply to the fourth deep lumbrical (4DL) muscle in adult rats. Both the lateral plantar nerves (LPN) and sural nerves (SN) supply axons to the 4DL muscle. The LPN normally supplies about ten motor axons that innervate more than 70% of the 4DL muscle fibers; the SN normally provides one to three motor axons that supply less than 30% of the muscle fibers[19]. Injury to the SN, therefore, results in minor partial denervation of the 4DL. Relatively little reactive sprouting by each LPN axon is required to bring about complete collateral reinnervation of SN-denervated muscle fibers. Thus, within about a week of SN crush, almost all fibers are innervated by the LPN[18] (see below). Following regeneration of SN motor axons, a very small fraction (less than 5%) of the reinnervated 4DL motor endplates acquire terminals supplied by both nerves. Most of these polyneuronally innervated muscle fibers become mononeuronally innervated by an axon in one or other of the two nerves, through competitive synapse elimination.

Here we asked whether any reinnervated 4DL muscle fibers would become either mainly or exclusively supplied by regener-

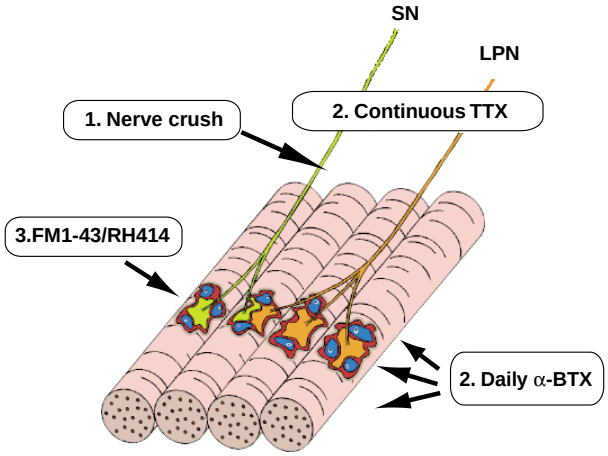

Fig. 1. Experimental design. First (1), 4DL muscles were subjected to bilateral minor partial denervation by crushing of the sural nerve (SN). Sprouting of intact lateral plantar nerve (LPN) axons and their terminals brought about effective collateral reinnervation of almost all muscle fibers within 14 d. Regenerating SN axons returned after about 15 d, polyneuronally innervating muscle fibers supplied by the LPN. Next (2), from 14 d after SN crush, sciatic nerve conduction and synaptic transmission in 4DL were blocked using tetrodotoxin (TTX) and α-BTX. Finally (3), styryl dyes were used to label SN (green; FM1-43) and LPN (orange; RH414) motor nerve terminals in isolated preparations. Blue, terminal Schwann cells at endplates. Fluorescence microscopy established the proportions of SN, LPN and dual innervation.

ating SN motor axons when all neuromuscular activity was blocked. If activity were necessary for competitive synapse elimination, then no synapse elimination would occur in paralyzed muscles; all muscle fibers reinnervated by the SN should also retain their sprouted LPN inputs (Fig. 1). However, if neuromuscular activity were not required, then there would be evidence of exclusive or majority innervation of endplates by the SN despite complete neuromuscular paralysis.

RESULTS

We assessed the innervation patterns of reinnervated 4DL muscles by staining SN and LPN motor nerve terminals with the vital fluorescent styryl dyes FM1-43 and RH414, respectively[12,20] (Fig. 2). The assays were carried out after two weeks of continuous, complete nerve conduction block, with or without additional neuromuscular block. During reinnervation of chronically paralyzed muscles, regenerating SN synapses competitively displaced LPN synapses from most polyneuronally innervated muscle fibers. Control experiments established that LPN sprouting was nearly complete by the time regenerating SN axons returned, that chronic paralysis was complete throughout the period of SN regeneration and that there was no direct toxic effect of either tetrodotoxin (TTX) or α-bungarotoxin (α-BTX) on motor nerve terminals.

Chronic nerve block did not prevent synapse elimination

The first signs of reinnervation of partially denervated 4DL by SN motor axons were observed no earlier than 15 days after nerve crush[11,14,18]. When propagated neuromuscular activity alone was blocked by chronic superfusion of the sciatic nerve of one hind limb with tetrodotoxin (TTX only), 10.1 ± 2.3% (mean ± s.e.m.) of muscle fibers became polyneuronally innervated by SN and LPN terminals (n = 399 fibers in four muscles; Fig. 3a). A further 4.9 ± 1.7% became reinnervated exclusively by regenerating SN terminals alone. Thus about 15% of total muscle fibers reacquired SN input, that is, about half the number in most unoperated muscles. The area occupied by SN motor nerve terminals at the polyneuronal endplates in chronic TTX-only muscles (107.2 ± 9.3 μm^2, mean ± s.e.m.; n = 41 endplates in four muscles) was significantly greater than that of intact LPN terminals supplying the same junctions (56.0 ± 6.5 μm^2; $p < 0.0001$, Mann-Whitney test; Fig. 3b). The total area of the polyneuronally innervated motor endplates in the TTX-only muscles was not discernibly different from that of the mononeuronally innervated junctions. Thus, the overwhelming majority of fibers innervated by both LPN and SN were more than 50% occupied by regenerating SN terminals, and this feature was independent of the overall sizes of the endplates (Fig. 3c).

Synapse elimination continued during neuromuscular block

It was possible that spontaneous quantal or non-quantal release and action of neurotransmitter at the endplate[21] could have been involved in competition between intact and regenerating synaptic terminals. To test this, we blocked all acetylcholine-induced activity at 4DL neuromuscular junctions by combining chronic TTX superfusion of the sciatic nerve, as described above, with daily local injections of α-BTX (Fig. 1). This procedure ensured complete block of all extant and newly synthesized acetylcholine receptors in the hind foot musculature[22] (checks described below). After two weeks, muscles were isolated, and LPN and SN terminals were stained with the vital dyes as in the TTX-only group (Fig. 2).

About 15% of the motor endplates in these totally paralyzed muscles (128 of 804 fibers in five muscles) also became reinnervated by regenerating SN motor axons (Fig. 3d). The percentage of polyneuronal innervation was 13.1 ± 4.5%, whereas 4.0 ± 1.0% of total fibers were exclusively supplied by regenerated, FM1-43-stained SN synaptic boutons. All five muscles contained examples of these mononeuronal, SN-innervated endplates. The distributions of motor nerve terminal areas on polyneuronally innervated muscle fibers were about equal (Fig. 3e). Moreover, the distribution of percentage occupancies was not discernibly biased toward either nerve (Fig. 3f). The mean fractional reoccupancy by SN terminals at polyneuronally innervated junctions was significantly smaller than that in the TTX-only or crush-only control groups ($p < 0.05$, ANOVA; Fig. 3f). Complete neuromuscular block might therefore have introduced a slight additional delay in competitive synapse elimination. However, competition was clearly not prevented by either form of paralysis. As almost all motor endplates were fully occupied by LPN terminals at the time regenerating SN axons returned (see below), competitive displacement of LPN synaptic boutons by SN boutons must have occurred at most, if not all, of the paralyzed endplates supplied by the two nerves.

By contrast, in reinnervated control muscles that were not blocked (crush-only controls), only about 5% of total muscle fibers had become reinnervated by the SN by 30 days after nerve crush (Fig. 3g), even though the SN axons probably regenerated down their original endoneurial tubes and back to their original endplates[23]. There were few polyneuronally innervated fibers in these muscles (2.3 ± 0.8% of 595 fibers in five muscles). Thus, about half of the endplates supplied by regenerated SN axons had already become mononeuronally innervated (1.9 ± 0.4% of total fibers). The distribution of fractional occupancies in the small number of polyneuronally innervated junctions that remained was similar to that in the TTX-only group (Fig. 3h and i).

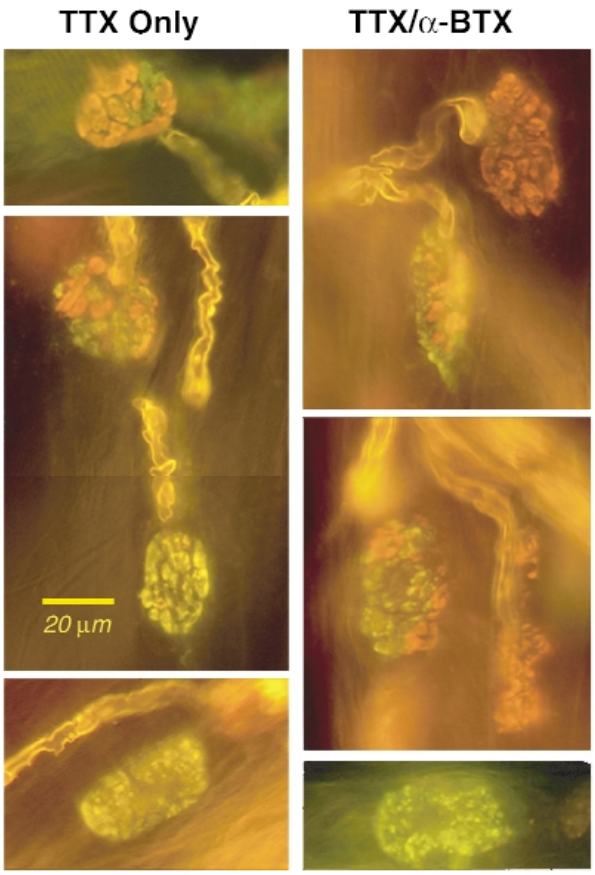

Fig. 2. Neuromuscular junctions in paralyzed muscles stained with vital styryl dyes. SN-reinnervated muscles after TTX block of nerve conduction alone (TTX only) or complete paralysis (TTX/α-BTX). LPN terminals were stained fluorescent orange by stimulating the nerve in isolated preparation in the presence of RH414; SN terminals were stained fluorescent green/yellow in similar way with FM1-43. Endplates were supplied by LPN axons alone (orange boutons only), by dual innervation with variable fractions of the endplate supplied by either nerve, or by regenerating SN terminals alone (green/yellow boutons). Motor axons often appeared fluorescent yellow in these preparations, because of passive (activity-independent) staining of myelin by the successive application of the two styryl dyes.

Together, these measurements suggest that nerve conduction and neuromuscular transmission block, and the consequent muscle paralysis, promoted sprouting of the LPN and regeneration of the SN motor axons and their terminals. This evidently increased the opportunities for SN terminals to competitively displace synaptic boutons belonging to intact LPN terminals. Thus, muscle paralysis measurably increased the proportion of muscle fibers that became reinnervated by the regenerating SN motor axons, restoring about half the original complement of SN motor nerve terminals; however, synapse elimination continued at a significant subset of the neuromuscular junctions, despite the chronic nerve conduction and neuromuscular blocks.

Sprouting and paralysis were effective and complete

Correct interpretation of the data in Fig. 3 depends on three assumptions: that the endplates were fully or mostly occupied when the regenerating axons returned, that paralysis of the muscles was complete during reinnervation by the SN, and that there was no direct toxicity or mechanical trauma caused by administration of either TTX or α-BTX to motor axons or their terminals. We carried out several checks to address these issues.

Previous electrophysiological data[18] seemed to rule out the possibility that the regenerating axons merely reoccupied junctions that failed to become innervated by sprouts from LPN axons and their terminals, but to examine this further, we vitally stained seven muscles with FM1-43 and α-BTX fluorescently labeled with tetramethylrhodamine isothiocyanate (TRITC–α-BTX) 14 days after the SN was cut. Between 200 and 400 endplates were examined in each muscle. No examples of unoccupied endplates were located; almost all endplates were fully occupied by LPN terminals (Fig. 4a). One endplate was about 50% occupied by an LPN terminal, but the fractional occupancy of all other endplates examined was greater than 80% of the total endplate area. Most of the partly occupied endplates showed only one or two vacant acetylcholine receptor clusters (Fig. 4b and c).

Styryl dyes like FM1-43 and RH414 label terminals by staining recycling synaptic vesicles; therefore, the dyes do not label nerve sprouts very well. However, other muscles were immunostained for neurofilament/SV2 and with TRITC–α-BTX to visualize acetylcholine receptors 3–14 days after the SN was crushed. Within three days, SN terminals had degenerated, and LPN axons and terminals had already begun to sprout, forming bridges between innervated and neighboring denervated muscle fiber endplates (data not shown)[24]. At 14 days after SN crush, we found only two examples of unoccupied endplates, of 1,483 fibers examined from thirteen muscles (Fig. 4d). An additional 51 endplates (3.4% of total examined) were partially occupied, but in all these, the LPN terminals occupied at least 80% of the endplate area. Thus, overall, the immunocytochemical data confirmed the vital staining, and indicated that LPN sprouting was nearly complete within 14 days of SN injury. Less than 1% of all endplates remained vacant or partly vacant by the time regenerating SN axons returned to the 4DL muscles. A few muscles were also examined by immunostaining after regenerating axons had returned. There were neurofilament-positive sprouts linking endplates in all three groups of reinnervated muscles (Fig. 4e).

It was also important to be confident that the TTX/α-BTX-blocked muscles were completely paralyzed. Tests on isolated preparations, with daily behavioral tests of neuromuscular function *in vivo*, confirmed that almost all endplates were completely blocked. First, low-impedance NaCl-filled micropipettes were used to probe endplates for residual synaptic currents. No spontaneous or nerve-evoked synaptic currents could be elicited from the TTX/α-BTX-paralyzed muscles, whereas strong signals were evoked from the endplate region of reinnervated controls (Fig. 5a–d). Spontaneous activity due to endogenous muscle fiber fibrillation was also absent from the chronically blocked muscles, and no fibrillation potentials were detected in extracellular or intracellular muscle fiber recordings[25]. The amount of unlabeled α-BTX used for chronic injections, when added to medium bathing control muscles, rapidly (within about 10 min) produced complete block of neuromuscular transmission (Fig. 5d). Next, fluorescent TRITC–α-BTX was injected into both hind feet of one rat on the day before the acute experiment. Staining was absent from endplates on the side that had received daily injections of unlabeled toxin for the preceding 13 days, but receptors on the control side became heavily labeled with the fluorescent toxin (Fig. 5e and f). Finally, a saturating concentration of fluorescent TRITC–α-BTX was added to medium bathing two chronically paralyzed muscles on the day of the acute experiment. Less

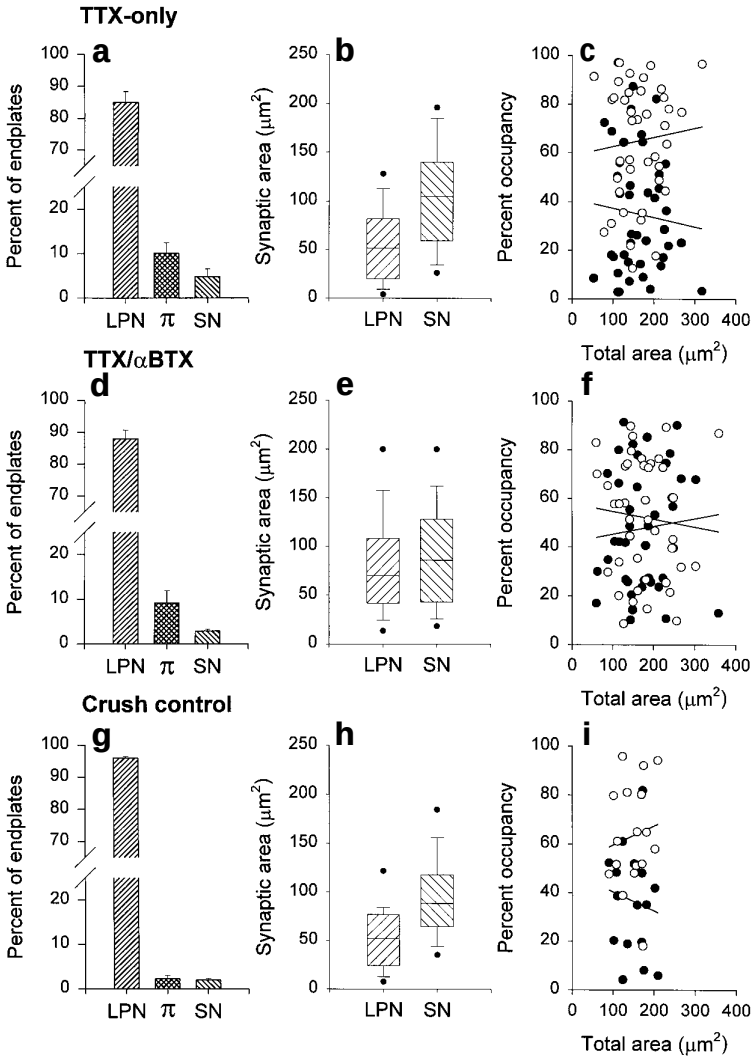

Fig. 3. Motor endplates in paralyzed muscles became mainly or exclusively reinnervated by inactive axons. Effects of nerve conduction block alone (TTX-only), of complete neuromuscular paralysis (TTX/αBTX), and of nerve crush without paralysis (crush control) on the pattern of reinnervation of 4DL muscles following partial denervation and SN regeneration. (**a**, **d**, **g**) Percentages of muscle fibers examined supplied by LPN axons alone, SN axons alone or combined (π) innervation. Bars represent s.e.m. (**b**, **e**, **h**) Box-whisker plots showing the distribution of areas of SN and LPN terminal boutons at endplates convergently innervated by axons in both nerves. Boxes, 25–75% interquartile range; horizontal lines in boxes, median area; whisker, 10–90% range; circles, 5% and 95% outliers. (**c**, **f**, **i**) Percent occupancies of endplates receiving inputs from both nerves (open circles, SN synapses; filled circles, LPN synapses), in relation to total synaptic area.

than 2% of endplates showed evidence (not shown) of any staining, and this comprised only faint punctate patches covering less than 50% of each endplate, whereas all endplates in the contralateral control pinned alongside in the same chamber were strongly stained. In four other muscles, BTX injections were curtailed one day early. Endplates were only faintly stained with TRITC–α-BTX a day later.

A residual concern was that administration of either TTX or α-BTX might have damaged some axons or their terminals, yielding vacant endplates that might have become either mono- or polyneuronally innervated by regenerating axons. Previous analysis indicated that the technique used to block nerve conduction neither mechanically nor chemically injured motor axons[11–14]. The α-BTX injections were made subcutaneously rather than intramuscularly, which also minimized the possibility of mechanical or chemical trauma to motor nerve terminals. However, to test the possibility of inadvertent damage by these treatments, we carried out a group of control experiments on four animals in which we first partially denervated the 4DL muscles by SN crush. Fourteen days later, TTX block was initiated as in the other experimental groups, but at the same time we deliberately resectioned the SN axons. Then we ascertained whether there were any denervated (vacant) endplates after a further 14 days of continuous TTX block, accompanied by daily injections of α-BTX. No uninnervated receptor patches were detected in FM1-43/TRITC–α-BTX-stained preparations of these muscles; that is, all the endplates seemed to be fully or mostly occupied by LPN motor nerve terminals (506 endplates examined in 4 muscles). As most endplates were also almost completely occupied by LPN axons at 14 days (Fig. 3), these control data are consistent with absence of traumatic injury of axons and terminals during the period of toxin administration.

DISCUSSION

The results from all the experimental and control groups together suggest that motor endplates became mostly or fully occupied by the SN motor terminals because intact LPN axons were competitively displaced by the regenerating inactive terminals, even though the postsynaptic sites at which this competition took place were unresponsive to acetylcholine and therefore completely electrically silent. Almost all endplates were more than 80% occupied by the time regenerating axons returned. As most SN axon terminals had reoccupied more than 20% of the endplate area, and there was no change in total endplate area, competitive displacement of LPN axons is the simplest and most reasonable explanation for the relative sizes of the two nerve inputs to these junctions.

The incidence of polyneuronal innervation was greater in chronically paralyzed muscles than in controls, consistent with previous findings[3,28]. This may be interpreted in terms of either a sprouting stimulus released by inactive muscle fibers promoting growth of the regenerating axons, and/or a stimulus from the paralyzed muscle fibers that inhibits or prevents synapse elimination. Thus, our previous data show that when the LPN is injured just before the SN returns to 4DL, many more muscle fibers are reinnervated by the SN than if the LPN is either intact or selectively blocked. However, more fibers become reinnervated than controls when nerve conduction is blocked, as we confirmed here using direct visualization with styryl dyes. Thus, the presence of LPN motor nerve terminals occupying motor endplates inhibits or represses synapse formation by regenerating SN axons, whether or not they are active[14]. Here the proportions of the reinnervated endplates that were recaptured by regenerating axons were, on average, greater after blocking nerve conduc-

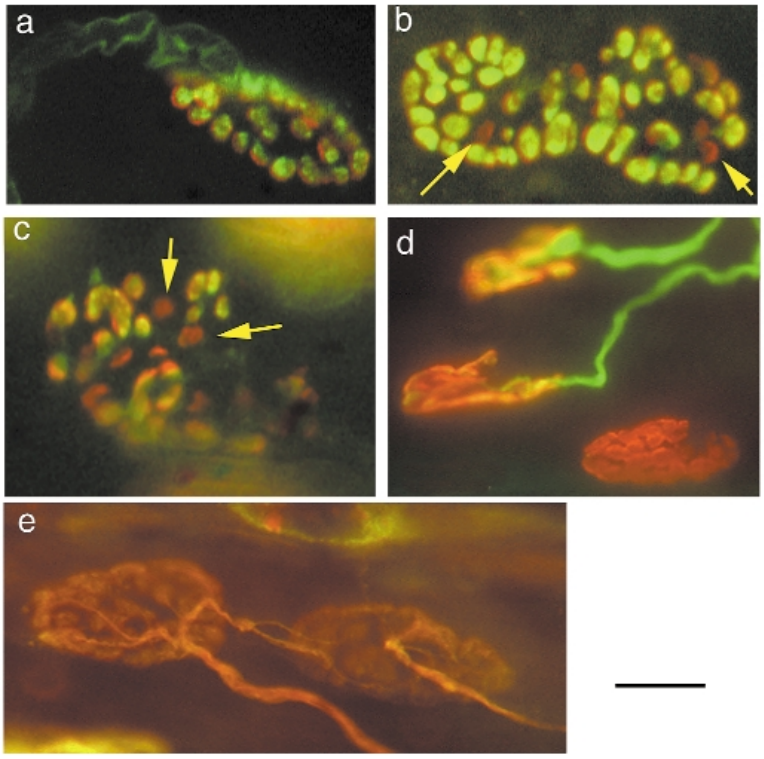

Fig. 4. Control experiments showing that almost all muscle fibers were occupied by LPN terminals or their sprouts before the return of regenerating SN axons. (**a**–**c**) Preparations in which acetylcholine receptors (red) were stained with TRITC–α-BTX and remaining LPN axon terminals (green) were stained with FM1-43, 14 d after nerve crush. (**a**) Fully occupied neuromuscular junction in which each receptor patch is covered by an FM1-43-stained LPN synaptic bouton. More than 95% of endplates were fully occupied in this way in the partially denervated muscles at 14 d. (**b**, **c**) Occasionally, endplates partially occupied by LPN terminals were found. Small patches of acetylcholine receptors stained with fluorescent TRITC–α-BTX (arrows) were not covered by overlying synaptic boutons at these endplates. (**d**) Neuromuscular junction 14 d after nerve crush in a preparation stained immunocytochemically for neurofilament, SV2 and TRITC–α-BTX to visualize axons, synaptic boutons and motor endplates, respectively. The endplate at the bottom was one of very few encountered that was not occupied by sprouts from nearby intact LPN motor axons and their terminals. (**e**) Immunostaining of a pair of endplates interconnected by sprouts and regenerating axons in a preparation examined after 14 d of neuromuscular paralysis (TTX/BTX), 28 d after SN crush. Scale bar, 10 μm (**a**–**c**); 20 μm (**d**, **e**).

tion alone. Thus, spontaneous activity at motor endplates may confer a slight competitive advantage on regenerating axons. However, this does not detract from the competitive success of regenerating axons in the completely paralyzed muscles. Significant numbers of endplates acquired exclusive innervation by regenerated boutons in the paralyzed muscles, and most endplates were also more than 20% reoccupied by SN boutons. This should not have occurred if modulation of postsynaptic activity were essential for synapse elimination. Thus, our experiments provide the most compelling evidence so far that activity and, more specifically, activation of acetylcholine receptors is not required for competitive elimination of mammalian neuromuscular synapses.

These findings contrast with other reports that synapse elimination only occurs from endplates that are partly, but not completely, blocked with α-BTX[15]. Perhaps the main difference in our experimental design was that axons were compelled to vie for occupancy of their former postsynaptic sites. The alternative, that the original postsynaptic sites were removed and replaced by new ones induced by the regenerating axons, is not consistent with either the persistence of endplate morphology after complete denervation in adults[15,26] or our finding that total endplate area was the same in paralyzed and crush-only muscles (Fig. 3). Furthermore, our findings are complementary to the results of other, developmental studies. For example, synapse formation and elimination occur normally in mutant zebrafish lacking nicotinic acetylcholine receptors[27].

Our findings may also be compared with synaptic plasticity in other parts of the nervous system. For example, changes in strength or stability are not restricted to active synapses during long-term potentiation in hippocampal neurons[7,8]. Related studies of connectivity in the visual system show that organization of orientation columns, although sensitive to activity, may occur without exposure to contoured visual stimuli[4,6], and ocular dominance columns can form without retinal input, and therefore retinal activity[5]. Our study has shown, at the level of individual identified synapses, that activity, although facilitating synaptic competition, is not obligatory for synapse elimination, and that ligand gating of postsynaptic receptors is not an essential, instructive step in the mechanism of the competition at most endplates.

Previous findings suggest that in addition to delaying synapse elimination, selective nerve conduction block confers a competitive advantage on active terminals and synaptic boutons over inactive ones[13–15]. Although these previous data may seem at first inconsistent with our findings here, one possibility is that activity is one of many permissive influences in synaptic competition, and the instructive selection of which synapses persist is governed by other factors. It is well established that paralysis of muscle stimulates motor nerve growth (sprouting)[2,3,21] and promotes retention of polyneuronal innervation[9,11,28]. The simplest and most conventional explanation is that this is due to production of growth factors by muscle fibers[36]. Thus, axons regenerating into the paralyzed muscles, as in our experiments here, may have responded better to strong growth-promoting stimuli (growth factors) released from the paralyzed muscle fibers, by virtue of higher levels of expression of receptors for these factors in the nerve terminal membranes. Intact terminals or their sprouts already occupying motor endplates might have expressed fewer receptors, decreasing their competitiveness. Computational analysis of a model in which neurotrophic factors are presumed to induce the expression of additional presynaptic receptors for these molecules predicts a range of experimental findings, including those here[30].

Further experiments using paralyzed, reinnervated muscles as an example may establish the primary molecular resources underpinning neuromuscular synaptic competition and whether these mediate a consumptive or a spatial form of biological competition[28,29]. Regenerating, but not intact, motor nerve terminals in adult muscles undergo reactive growth in response to glial cell line-derived neurotrophic factor (GDNF)

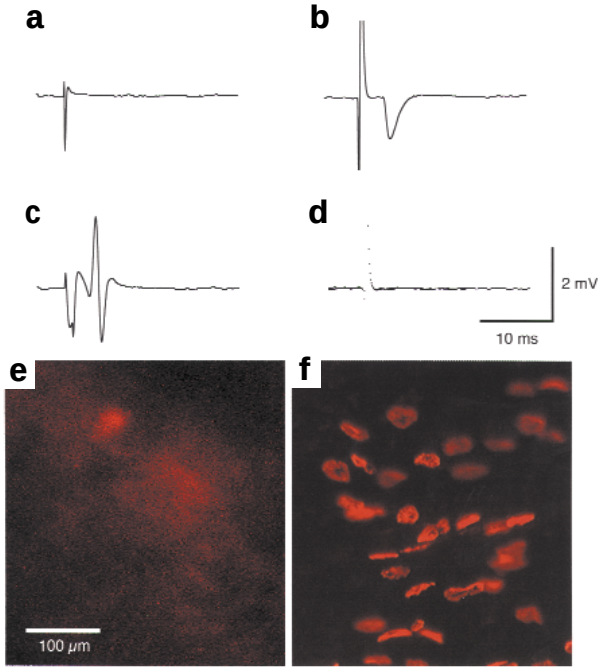

Fig. 5. Control experiments confirming the effectiveness of the chronic neuromuscular block. (**a**–**d**) Electrophysiological recordings from paralyzed (**a**, **c**) and reinnervated control (**b**, **d**) 4DL muscles. (**a**) Focal EMG recording showing no response to nerve stimulation in a muscle paralyzed for 13 d. (**b**) Extracellular endplate current following indirect stimulation of the muscle nerve to the contralateral control muscle; the muscle was pre-incubated with 2 μM μ-conotoxin to block muscle action potentials. (**c**) Action currents in the paralyzed muscle following direct stimulation of muscle fibers. (**d**) Abolition of the synaptic current in the control muscle after the addition of unlabeled α-BTX to the bathing solution. (**e**, **f**) Fluorescent images in the vicinity of intramuscular nerves, from chronically paralyzed muscles (**e**) and control muscles (**f**) in the same rat 24 h after injection of fluorescent TRITC–α-BTX into both hind feet. There was no staining on the chronically paralyzed side, indicating the receptors were all occupied by unlabeled toxin.

overexpression (M.L. Bernstein *et al.*, *Soc. Neurosci. Abstr.* 24, 413.3, 1998), and other studies suggest that in immature muscle, GDNF sustains high levels of polyneuronal innervation[31] and enhances synaptic transmission[32]. An alternative kind of mechanism is that the regenerating terminals compete effectively by more direct interference[28,29], possibly mediated by a balance between secreted proteases and endogenous protease inhibitors[33,34]. In this regard, although we blocked the action of neurotransmitter on postsynaptic membranes, we did not prevent spontaneous exocytosis of neurotransmitter or other molecules from the competing presynaptic terminals. Exocytosis of small molecules and peptides may be independently regulated in neural cells[35]. The function of exocytosis–endocytosis in competition for neurotrophic molecules or endplate space could be tested by blocking all vesicle recycling during nerve regeneration with botulinum toxin, for example, although different methods would be required to assess the outcome, as styryl dyes require vesicle recycling to produce selective vital staining of nerve terminals.

Resumption of activity after chronic nerve conduction block is not sufficient to bring about synapse elimination at all reinnervated neuromuscular junctions[37]. Our study here showed that activity is not even necessary for neuromuscular synapse elimination. Whereas other studies have shown that when activity is present, remodeling of neuromuscular synapses occurs, our study showed that when activity was absent, there was still remodeling of synaptic connections. Whether paralysis stimulates remodeling by promoting both sprouting and withdrawal of terminal arbors remains an open question. However, our results emphasize the idea that activity is influential but not decisive in the competition for exclusive innervation of motor endplates, and that the function of activity may be more permissive than instructive[12]. Finally, if inactivity leads to similar reactive growth of axons and enhancement of the opportunities for competitive restoration of connections elsewhere in the nervous system, then a period of synaptic silence, imposed locally at sites of injury, might actually promote regeneration and restoration of functional connections in these regions also.

METHODS

All experiments were carried out under license in accordance with UK Home Office regulations. Adult female Sprague-Dawley rats were deeply anesthetized by inhalation of halothane/nitrous oxide. In the first experimental group, the SN was crushed with fine forceps, then 14 d later the animals were re-anesthetized, and Alzet osmotic minipumps (Charles River, Margate, UK), containing tetrodotoxin (TTX; Calbiochem, San Diego, California, or Sigma, Poole, UK) at a concentration of 500 μg per ml in 0.9% saline with antibiotics, were implanted. The pumps were connected by subcutaneous tubing to the inside of a cuff placed around the sciatic nerve, as described[12,18]. The advantage of this technique is that it does not cause nerve damage because the cuffs are loose-fitting and merely secure the tip of the indwelling cannula to the vicinity of the nerve, while restricting the rapid diffusion of TTX from its site of administration and thereby ensuring patent, reliable and long-lasting nerve conduction block. The distal hind limb remained completely unused during the period of chronic nerve conduction block. Toe extension and flexion withdrawal reflexes were tested daily and were routinely absent for at least two weeks. These signs are reliable indicators of complete and continuous nerve conduction block[18]. In the second group, all transmitted activity at endplates was abolished by combining reinnervation and nerve block as described above. In addition, the rats were lightly anesthetized with halothane each day (including the day of the acute experiment), and the hind foot pad on the TTX-blocked side was injected subcutaneously over the lumbrical muscles with 5 μl purified, unlabeled α-BTX (Molecular Probes, Leiden, Netherlands), at a concentration of 500 μg per ml, to block all extant and newly synthesized acetylcholine receptors. The α-BTX was dissolved in a buffer solution of 20 mM sodium acetate, 100 mM arginine, 1% mannitol, 100 mM sodium sulfate and 0.1% bovine serum albumin, pH 6.9.

Only animals with persistent absence of reflexes were studied further. In some animals, TRITC–α-BTX (Molecular Probes) was injected into the foot either on the last or the penultimate day of the chronic experiment. After 14 d of nerve and/or neuromuscular block, 4DL preparations with attached SN and LPN nerves were isolated (removing the TTX block). In some cases, extracellular recordings were made from the endplate regions with standard electrophysiological techniques, using micropipettes filled with 1 M NaCl and broken at their tips to yield a tip resistance of 1–10 MΩ. Recordings from contralateral reinnervated muscles were made after 20 min of incubation in 2 μM μ-conotoxin (Scientific Marketing Associates, Barnet, UK) to selectively block muscle action potentials and abolish contraction. This treatment was unnecessary for muscles that had received chronic α-BTX treatment because they did not twitch in response to nerve stimulation.

Motor nerve terminals supplied by the SN and LPN were identified by selective loading with 4–8 μM FM1-43 and 25 μM RH414 (both from Molecular Probes). In most experiments, muscles were first bathed in FM1-43, and the SN terminals were stained by stimulating the nerve at 20 Hz continuously for 10 min. After muscles were washed for 15–20 min, RH414 was added, and the LPN terminals were stained using a similar pattern of stimulation. This procedure rendered SN boutons fluorescent

green/yellow and LPN boutons orange when viewed with a fluorescence filter cube with 435 nm excitation, 455 nm dichroic mirror and 515IF emission filter. Mononeuronal and polyneuronal innervation were assigned scores using fluorescence microscopy (Fig. 2). Control experiments showed that terminals and synaptic boutons only stained with the dyes when the axons were stimulated. Thus, for example, stimulation of one of the axons to a polyneuronally innervated endplate in the presence of FM1-43 stained its boutons fluorescent green, whereas incubation in RH414 without stimulation produced no orange staining of either the remaining boutons or any of the boutons at mononeuronally innervated endplates. The two dyes, when used selectively to stain SN and LPN synaptic boutons, therefore accurately revealed the extent of innervation of individual endplates by these motor axons. Endplate areas and fractional occupancies were measured from binary masks constructed to exactly overlay areas stained with FM1-43 or RH414, using Openlab software (Improvision, Coventry, UK). LPN terminals in muscles denervated for 14 d were vitally stained with FM1-43 by bathing the muscles for 5–10 min in a depolarizing solution of the dye, in which potassium ion concentration was increased to 50 mM, with the sodium ion concentration reduced by 45 mM. Immunocytochemical staining was carried out by fixing 4DL muscles for 15 min in 4% paraformaldehyde in phosphate-buffered saline. Muscles were then incubated for 20 min in 5 µg per ml TRITC–α-BTX to label junctional acetylcholine receptors, and were permeabilized in methanol at –20ºC for 7 min. This was followed by labeling of axons and nerve terminals with antibodies against the 165-kDa neurofilament protein (diluted 1:250) and the synaptic vesicle antigen SV2 (diluted 1:500). Both primary antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, Iowa). Antibody binding was visualized using fluorescein isothiocyanate-conjugated secondary antibodies. In some of the preparations, there were areas where either primary or secondary antibodies had evidently failed to penetrate very well to the deepest fibers, yielding only weak fluorescence. Thus our observations focused mainly on superficial fibers on both sides of the muscles.

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1. Lohof, A. M., Delhaye-Bouchaud, N. & Mariani, J. Synapse elimination in the central nervous system: functional significance and cellular mechanisms. *Rev. Neurosci*. 7, 85–101 (1996).
2. Grinnell, A. D. Dynamics of nerve-muscle interaction in developing and mature neuromuscular junctions. *Physiol. Rev*. 75, 789–834 (1995).
3. Purves, D. *Neural Activity and the Growth of the Brain* (Cambridge Univ. Press, Cambridge, UK, 1994).
4. Crair, M. C., Gillespie, D. C. & Stryker, M. P. The role of visual experience in the development of columns in cat visual cortex. *Science* 279, 566–570 (1998).
5. Crowley, J. C. & Katz, L. C. Development of ocular dominance columns in the absence of retinal input. *Nat. Neurosci.* 2, 1125–1130 (1999).
6. Sengpiel, F., Stawinski, P. & Bonhoeffer, T. Influence of experience on orientation maps in cat visual cortex. *Nat. Neurosci.* 2, 727–732 (1999).
7. Engert, F. & Bonhoeffer, T. Synapse specificity of long-term potentiation breaks down at short distances. *Nature* 388, 279–284 (1997).
8. Fitzsimonds, R. M., Song, H. J. & Poo, M. M. Propagation of activity-dependent synaptic depression in simple neural networks. *Nature* 388, 439–448 (1997).
9. Sanes, J. R. & Lichtman, J. W. Development of the vertebrate neuromuscular junction. *Annu. Rev. Neurosci.* 22, 389–442 (1999).
10. Brown, M. C., Jansen, J. K. S. & Van Essen, D. C. Polyneuronal innervation in newborn rats and its elimination during maturation. *J. Physiol. (Lond.)* 261, 387–422 (1976).
11. Ribchester, R. R. Activity-dependent and activity-independent synaptic interactions during reinnervation of partially denervated rat muscle. *J. Physiol. (Lond.)* 401, 53–75 (1988).
12. Barry, J. A. & Ribchester, R. R. Persistent polyneuronal innervation in partially denervated rat muscle after reinnervation and recovery from prolonged nerve-conduction block. *J. Neurosci*. 15, 6327–6339 (1995).
13. Ribchester, R. R. & Taxt, T. Motor unit size and synaptic competition in rat lumbrical muscles reinnervated by active and inactive motor axons. *J. Physiol. (Lond.)* 344, 89–111 (1983).
14. Ribchester, R. R. & Taxt, T. Repression of inactive motor-nerve terminals in partially denervated rat muscle after regeneration of active motor axons. *J. Physiol. (Lond.)* 347, 497–511 (1984).
15. Balice-Gordon, R. J. & Lichtman, J. W. Long-term synapse loss induced by focal blockade of postsynaptic receptors. *Nature* 372, 519–524 (1994).
16. Brown, M. C., Hopkins, W. G. & Keynes, R. J. Short-term and long-term effects of paralysis on the motor innervation of 2 different neonatal mouse muscles. *J. Physiol. (Lond.)* 329, 439–450 (1982).
17. Callaway, E. M., Soha, J. M. & Van Essen, D. C. Differential loss of neuromuscular connections according to activity level and spinal position of neonatal rabbit soleus motor neurons. *J. Neurosci*. 9, 1806–1824 (1989).
18. Ribchester, R. R. Coexistence and elimination of convergent motor-nerve terminals in reinnervated and paralyzed adult rat skeletal muscle. *J. Physiol. (Lond.)* 466, 421–441 (1993).
19. Betz, W. J., Caldwell, J. H. & Ribchester, R. R. The size of motor units during post-natal development of rat lumbrical muscle. *J. Physiol. (Lond.)* 297, 463–478 (1979).
20. Betz, W. J., Mao, F. & Bewick, G. S. Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *J. Neurosci.* 12, 363–375 (1992).
21. Vyskocil, F. & Vrbova, G. Nonquantal release of acetylcholine affects polyneuronal innervation on developing rat muscle fibers. *Eur. J. Neurosci*. 5, 1677–1683 (1993).
22. Shyng, S. L. & Salpeter, M. M. Effect of reinnervation on the degradation rate of junctional acetylcholine receptors synthesized in denervated skeletal muscles. *J. Neurosci.* 10, 3905–3915 (1990).
21. Brown, M. C., Holland, R. L. & Hopkins, W. G. Motor nerve sprouting. *Annu. Rev. Neurosci.* 4, 17–42 (1981).
24. Son, Y. J., Trachtenberg, J. T. & Thompson, W. J. Schwann cells induce and guide sprouting and reinnervation of neuromuscular junctions. *Trends Neurosci*. 19, 280–285 (1996).
25. Purves, D. & Sakmann, B. Membrane properties underlying spontaneous activity of denervated muscle fibres. *J. Physiol. (Lond.)* 239, 125–153 (1974).
26. Rich, M. M. & Lichtman, J. W. In vivo visualization of pre- and postsynaptic changes during synapse elimination in reinnervated mouse muscle. *J. Neurosci*. 9, 1781–1805 (1989).
27. Liu, D. W. C. & Westerfield, M. The formation of terminal fields in the absence of competitive interactions among primary motoneurons in the zebrafish. *J. Neurosci.* 10, 3947–3959 (1990).
28. Ribchester, R. R. & Barry, J. A. Spatial versus consumptive competition at polyneuronally innervated neuromuscular junctions. *Exp. Physiol*. 79, 465–494 (1994).
29. Keddy, P. *Competition* (Chapman & Hall, London, New York, 1989).
30. Van Ooyen, A. & Willshaw, D. J. Competition for neurotrophic factor in the development of nerve connections. *Proc. R. Soc. Lond. B* 266, 883–892 (1999).
31. Nguyen, Q. T., Parsadanian, A. S., Snider, W. D. & Lichtman, J. W. Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. *Science* 279, 1725–1729 (1998).
32. Ribchester, R. R, Thomson, D., Haddow, L. J. & Ushkaryov, Y. A. Enhancement of spontaneous transmitter release at neonatal mouse neuromuscular junctions by the glial cell line-derived neurotrophic factor (GDNF). *J. Physiol. (Lond.)* 512, 635–641 (1998).
33. Zoubine, M. N., Ma, J. Y., Smirnova, I. V., Citron, B. A. & Festoff, B. W. A molecular mechanism for synapse elimination: novel inhibition of locally generated thrombin delays synapse loss in neonatal mouse muscle. *Dev. Biol*. 179, 447–457 (1996).
34. Chang, Q. & Balice-Gordon, R. J. Nip and tuck at the neuromuscular junction: a role for proteases in developmental synapse elimination. *Bioessays* 19, 271–275 (1997).
35. Angleson, J. K., Cochilla, A. J., Kilic, G., Nussinovitch, I. & Betz, W. J. Regulation of dense core release from neuroendocrine cells revealed by imaging single exocytic events. *Nat. Neurosci*. 2, 440–446 (1999).
36. Snider, W. D. & Lichtman, J. W. Are neurotrophins synaptotrophins. *Mol. Cell. Neurosci*. 7, 433–442 (1996).
37. Costanzo, E. M., Barry, J. A. & Ribchester, R. R. Co-regulation of synaptic efficacy at stable polyneuronally innervated neuromuscular junctions in reinnervated rat muscle. *J. Physiol. (Lond.)* 521, 365–374 (1999).